N-Acetyltransferase 2 Phenotype but not *NAT1*10* Genotype Affects Aminobiphenyl-Hemoglobin Adduct Levels[1]

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Abstract

Aminobiphenyls (ABPs) in tobacco have been implicated in bladder cancer etiology in smokers. *N*-Acetylation of ABPs in the liver, predominantly by the *N*-acetyltransferase 2 (NAT2) isozyme, represents a detoxification pathway, whereas *O*-acetylation of *N*-hydroxy-ABPs in the bladder, predominantly by the *N*-acetyltransferase 1 (NAT1) isozyme, represents a bioactivation pathway. We and others have demonstrated that NAT2 phenotype affects 3- and 4-ABP-hemoglobin adduct levels (higher levels in slow acetylators), which are considered valid biomarkers of the internal dose of ABP to the bladder. We have also shown that *NAT1* genotype (*NAT1*10* allele) is associated with increased DNA adduct levels in urothelial tissue and higher risk of bladder cancer among smokers. It is not known whether *NAT1*10* genotype influences ABP-hemoglobin adduct levels. Therefore, we assessed 403 primarily non-Hispanic white residents of Los Angeles County for their NAT2 acetylator phenotype, *NAT1*10* acetylator genotype, and 3- and 4-ABP-hemoglobin adduct levels. Eighty-two subjects were current tobacco smokers of varying intensities. Tobacco smokers had significantly higher mean 3- and 4-ABP-hemoglobin adduct levels relative to nonsmokers. The levels increased with increased amounts smoked per day (two-sided, $P < 0.0001$ in all cases). With adjustment for *NAT1* genotype and race, the smoking-adjusted geometric mean level of 3-ABP-hemoglobin adducts in NAT2 slow acetylators was 47% higher than that in NAT2 rapid acetylators ($P = 0.01$). The comparable value for 4-ABP-hemoglobin adducts was 17% ($P = 0.02$). In contrast, no association between *NAT1*10* genotype and 3- or 4 ABP-hemoglobin adduct levels was observed after adjustment for NAT2 phenotype, smoking, and race. The present study suggests that the impact of the *NAT1*10* genotype on 3- and 4-ABP-hemoglobin adducts is noninformative on the possible association between NAT1 activity and bladder cancer risk.

Introduction

Strong epidemiological and experimental data implicate ABPs[3] in the etiology of bladder cancer. 3- and 4-ABP are present in tobacco smoke in small quantities and are considered putative tobacco carcinogens responsible for bladder cancer development among smokers (1). ABPs require bioactivation before their metabolites can bind to DNA. In the liver, *N*-acetylation of ABPs (a detoxification step) competes with *N*-hydroxylation by cytochrome P450 1A2 (a bioactivation step). The metabolically active form of the arylamine (the *N*-hydroxylated amine) is a direct-acting mutagen and can form adducts with Hb and/or circulate freely or as a glucuronide conjugate and be excreted through the kidney (2). When the *N*-hydroxylated amine reaches bladder epithelial cells, it can bind directly to DNA or can be further bioactivated into a highly reactive species by *O*-acetylation (3). *N*-Acetylation of ABPs and *O*-acetylation of *N*-hydroxy-ABPs, respectively, are catalyzed by two related *N*-acetyltransferases, NAT2 and NAT1. NAT2 seems to be the predominant, but it may not be the exclusive enzyme responsible for *N*-acetylation in the liver because of its higher expression level (4). NAT1 has a wider tissue distribution and predominantly contributes to *O*-acetylation of *N*-hydroxy-ABPs in the human bladder (5). NAT2 and NAT1 enzyme activities, which both show wide interindividual variation, thus may modify ABP-associated bladder cancer risk.

A number of case-control studies have investigated the relationship between NAT2 acetylator pheno- or genotype and smoking-related bladder cancer risk. Overall, evidence suggests that NAT2 slow acetylators have an increased risk of bladder cancer (6). Talaska *et al.* (7) showed that DNA adduct levels in exfoliated bladder cells were lower in individuals with the NAT2 rapid phenotype.

Numerous polymorphisms in *NAT1* have been reported recently (8). Of particular interest is the *NAT1*10* allele, which has been associated with higher levels of DNA adducts in human bladder epithelial tissue (9). It has also been found to interact with *NAT2* genotype in increasing risk of bladder cancer among cigarette smokers (10, 11), a result that was not

Received 10/25/99; revised 3/8/00; accepted 3/27/00.
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[1] Supported by NIH Grants R35 CA53890, ROI [illegible] and P01 ES05622; by Grant 1RT-423 from the California Tobacco-related Disease Research Program; and by Grant 3233-054996.98/1 from the Swiss National Science Foundation.
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[3] The abbreviations used are: ABP, aminobiphenyl; Hb, hemoglobin; NAT1 and NAT2, *N*-acetyltransferase 1 and 2

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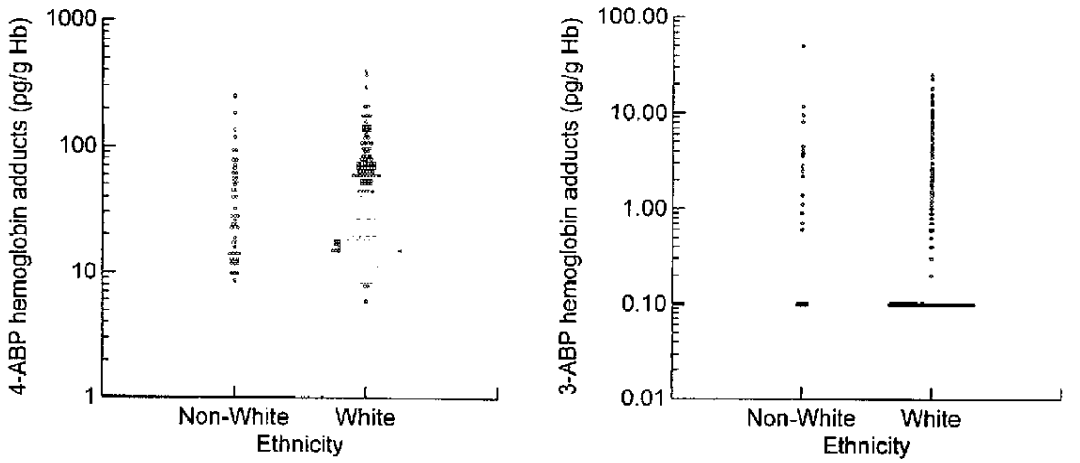

Fig. 1. Distribution of 3- and 4-ABP-Hb adducts among whites and non-whites.

confirmed by Okkels *et al.* (12). The mechanisms through which the *NAT1*10* genotype may influence either adduct levels or risk is uncertain, and these findings have yet to be confirmed by additional studies.

3- and 4-ABP-Hb adducts are considered valid biomarkers of the internal dose of ABP to the target organ (*i.e.*, bladder). An examination of ABP-Hb adducts *versus* DNA adducts in exfoliated urothelial cells of cigarette smokers and nonsmokers has shown a close correlation between levels of the two types of adducts (13). We and others have found higher 3- and 4-ABP-Hb adduct levels among slow NAT2 acetylators (1, 14), a result that is in concordance with the observed association between slow NAT2 activity and bladder cancer risk. The impact of NAT2 acetylator pheno- and genotype on 4-ABP-Hb adducts was found to be more prominent at low carcinogen exposure measured as cotinine and nicotine excreted into the 24-h urines of 50 smokers and 50 nonsmokers (15). To date, there is no information on the relationship between *NAT1* genotype and ABP-Hb adduct levels in humans. NAT1 has a wide extrahepatic tissue distribution. ABP-Hb adduct levels have the potential to reflect the impact of NAT1 activity on ABP metabolism in extrahepatic tissue, including bladder tissue, because ABP metabolites are transported throughout the body and can be reabsorbed from the bladder back into the circulation (2, 16). NAT1 is expressed in blood cells (17) and could potentially affect the formation of ABP-Hb adducts via *N*- or *O*-acetylation of ABP or *N*-hydroxy-ABP in erythrocytes. In other words, the extent to which ABP metabolism in nonhepatic tissues (such as principally NAT1-catalyzed *O*-acetylation) influences ABP-Hb adduct levels is unclear. The present study reports on the relationship between *NAT1*10* genotype and 3- and 4-ABP-Hb adduct levels in a population-based, primarily non-Hispanic white sample of healthy residents of Los Angeles County, California.

Materials and Methods

Subjects and Laboratory Tests. Four hundred three subjects were included in this study. The subjects were derived from two separate sources: (*a*) those who participated in a multiethnic survey described previously (14); and (*b*) control subjects for an ongoing case-control study of bladder cancer that used an identical protocol for urine and blood collection and included identical questions on current tobacco use as the multiethnic survey.

The multiethnic survey participants were male residents of Los Angeles County over the age of 35 years and were either non-Hispanic white ($n = 17$), black ($n = 10$), or Asian (Chinese or Japanese; $n = 14$). Twenty subjects were lifelong nonsmokers, whereas the remaining 21 subjects were current cigarette smokers of varying intensity.

The control subjects were the first 362 controls recruited for an ongoing case-control study of bladder cancer among non-Asian residents of Los Angeles County, ages 25–64 years. The case-control study was initiated immediately after the termination of the multiethnic survey. The subjects were either non-Hispanic white ($n = 337$), Hispanic white ($n = 21$), or black ($n = 4$). One hundred sixteen subjects were lifelong nonsmokers, and 185 subjects were exsmokers. The remaining 61 subjects were current tobacco smokers of varying intensity. Seventy-one (20%) of the 362 control subjects were women.

Thus, 403 subjects were included in this study; 88% were non-Hispanic white, and 82% were men. The mean age of the subjects was 58 years. Of the 82 current tobacco smokers, 69 were exclusive cigarette smokers and 8 were exclusive pipe/cigar smokers.

A blood specimen was obtained from each subject after an in-person interview during which the subject was asked about cigarette and other tobacco use during the past 60 days. Red cells extracted from whole blood were processed on the day of collection and then stored at −20°C until analysis for 3- and 4-ABP-Hb adduct levels (14). All study subjects had detectable levels of 4-ABP-Hb adducts. On the other hand, 3-ABP-Hb adducts were not detected in samples from 271 subjects. Because the detection limit of the ABP assay was 0.2 pg/g Hb, all subjects with nondetectable values were assigned an ABP-Hb adduct level that was midway between 0 and 0.2 (*i.e.*, 0.1 pg/g Hb).

DNA samples extracted from whole blood were stored at −70°C until *NAT1* genotyping. Genotyping was conducted as described previously for four published sequence variations in the 3′ region of *NAT1* near the putative polyadenylation signal, namely *NAT1*4* (most common allele), *NAT1*3*, *NAT1*10*, and *NAT1*11* (18). Because the *NAT1*10* allele, containing an

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Table 1 Geometric mean levels of 3- and 4-ABP-Hb adducts among study subjects

	White		Non-white	
	n	Mean (pg/g Hb)	*n*	Mean (pg/g Hb)
3-ABP-Hb adducts[a]				
Nonsmokers	288	0.30	33	0.45
1–19 cigarette-equivalents/day[b]	29	2.21	9	1.50
≥20 cigarette-equivalents/day	37	4.00	7	4.33
4-ABP-Hb adducts[a]				
Nonsmokers	288	20.1	33	21.9
1–19 cigarette-equivalents/day	29	49.5	9	50.7
≥20 cigarette-equivalents/day	37	72.7	7	91.2

[a] The effects of smoking intensity on 3- and 4-ABP-Hb adduct levels were statistically significant ($P < 0.0001$) in both ethnic groups.
[b] The tobacco contents of one cigar and one pipe were assumed to be equivalent to the tobacco contents of 4.5 and 2.5 cigarettes, respectively.

Table 2 Geometric mean levels of 3-ABP-Hb adducts in study subjects by NAT2 acetylator phenotype and *NAT1*10* genotype[a]

NAT2 phenotype	*NAT1*10* genotype					
	Non-*NAT1*10*		*NAT1*10*		Total	
	n	Mean (pg/g Hb)	*n*	Mean (pg/g Hb)	*n*	Mean (pg/g Hb)
Whites						
Slow	148	0.71	53	0.65	201	0.69[b]
Rapid	86	0.49	67	0.52	153	0.50[b]
Total	234	0.60[c]	120	0.59[c]		
All races						
Slow	162	0.88[d]	65	0.79[d]	227	0.84[e]
Rapid	91	0.59[d]	85	0.55[d]	176	0.57[e]
Total	253	0.73[f]	150	0.67[f]		

[a] Adjusted for number of cigarette-equivalents smoked per day. The tobacco contents of one cigar and one pipe were assumed to be equivalent to the tobacco contents of 4.5 and 2.5 cigarettes, respectively.
[b] Further adjusted for *NAT1* genotype: $P = 0.06$, NAT2 slow *vs.* rapid.
[c] Further adjusted for NAT2 phenotype: $P = 0.89$, non-*NAT1*10 vs. NAT1*10*.
[d] Further adjusted for race (white, non-white).
[e] Further adjusted for race and *NAT1* genotype: $P = 0.008$, NAT2 slow *vs.* rapid.
[f] Further adjusted for race and NAT2 phenotype: $P = 0.53$, non-*NAT1*10 vs. NAT1*10*.

altered polyadenylation signal, has been associated with elevated levels of DNA adducts and higher risk of bladder cancer (9, 10), genotypes containing at least one *NAT1*10* allele were compared with other, non-*NAT1*10* genotypes (*NAT1*10 versus* non-*NAT1*10* genotypes). The recently discovered, rare, and nonfunctional *NAT1* alleles (8) were not analyzed in this study.

In addition, each subject was asked to collect an overnight urine sample (ending with the first morning void) after drinking coffee prepared from two packets of Nescafe instant coffee (~70 mg of caffeine) between 3 and 6 p.m. the previous day. At the time of collection of the urine specimen, the subject was briefly interviewed about caffeine intake (in addition to the prescribed packets of instant coffee) and use of acetaminophen on the previous day. Excessive caffeine consumption (more than four cups of coffee) or acetaminophen use has the potential of affecting the validity of the phenotyping assay. All urine samples were acidified (20 mg ascorbic acid/ml urine) within 24 h of collection and subsequently were stored at −20°C until the NAT2 acetylator phenotype was determined using procedures described previously (14).

Statistical Analysis. The distributions of 3- and 4-ABP-Hb adduct levels in our study population were markedly skewed; therefore, formal statistical testing was performed on logarithmically transformed values of adduct levels, and geometric (as opposed to arithmetic) mean values are presented. We used the ANOVA method to compare ABP-Hb adduct levels by current level of tobacco smoking. The analysis of covariance method was used to assess the impact of NAT2 phenotype and *NAT1* genotype on ABP-Hb adduct levels while adjusting for the effect of smoking and race (white, non-white). All *P* values presented are two-sided (19).

Results

Thirteen subjects took acetaminophen-containing medication and 7 subjects had excessive caffeine consumption on the day prior to their overnight urine collection. Excluding these 20 subjects did not materially alter our findings; therefore, they were included in all analyses presented in this report. The distribution of 3- and 4-ABP-Hb adduct levels is presented separately for whites and non-whites in Fig. 1. The overall geometric mean levels of 3- and 4-ABP-Hb adducts in our study population were 0.61 and 25.5 pg/g Hb, respectively, in whites, and 0.93 and 31.3 pg/g Hb, respectively, in non-whites. There were statistically significant associations between levels of 3- and 4-ABP-Hb adducts and intensity of smoking in both ethnic groups ($P < 0.0001$ in both groups; Table 1).

Table 2 shows the smoking-adjusted geometric mean values of 3-ABP-Hb adducts by NAT2 phenotype and *NAT1*10* genotype for non-Hispanic white subjects only and for all subjects with adjustment for race (white, non-white). The mean was highest among subjects possessing both NAT2 slow and non-*NAT1*10* acetylator status (0.71 and 0.88 pg/g Hb in whites and all races, respectively). We did not observe an interactive effect of NAT2 phenotype and *NAT1*10* genotype on 3-ABP-Hb adduct levels ($P = 0.67$ and 0.87 in whites and all races, respectively). The observed variation in 3-ABP-Hb adduct levels between slow and rapid acetylators was primarily due to the impact of NAT2 phenotype. With adjustment for *NAT1*10* genotype, NAT2 slow acetylators exhibited mean 3-ABP-Hb adduct levels that were 38% ($P = 0.06$) and 47% ($P = 0.01$) higher than NAT2 rapid acetylators among white and all subjects, respectively. In contrast, there was no difference in mean 3-ABP-Hb adduct levels between *NAT1*10 versus* non-*NAT1*10* genotype either in whites ($P = 0.89$) or all subjects ($P = 0.53$) after adjustment for NAT2 phenotype and smoking (Table 2).

The relationship between 4-ABP-Hb adduct levels and NAT2/*NAT1*10* phenotype/genotype is presented in Table 3. No interactive effect of NAT2 phenotype and *NAT1*10* genotype on 4-ABP-Hb adduct levels was observed ($P = 0.97$ and 0.61 for whites and all races, respectively). Again, we noted a stronger effect of NAT2 phenotype than *NAT1*10* genotype on ABP-Hb adduct level after adjustment for level of cigarette smoking. Smoking-adjusted mean 4-ABP-Hb adducts were 16% higher in NAT2 slow than in NAT2 rapid acetylators after adjustment for *NAT1*10* genotype and race, and the difference was statistically significant ($P = 0.02$). *NAT1*10* genotype, on the other hand, showed no influence on smoking-adjusted 4-ABP-Hb adduct levels in either white only ($P = 0.99$) or all subjects ($P = 0.86$; Table 3).

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Table 3 Geometric mean levels of 4-ABP-Hb adducts in study subjects by NAT2 acetylator phenotype and *NAT1*10* genotype[a]

NAT2 phenotype	*NAT1*10* genotype					
	non-*NAT1*10*		*NAT1*10*		Total	
	n	Mean (pg/g Hb)	*n*	Mean (pg/g Hb)	*n*	Mean (pg/g Hb)
Whites						
Slow	148	26.7	53	26.7	201	26.7[b]
Rapid	86	24.0	67	24.1	153	24.0[b]
Total	234	25.3[c]	120	25.3[c]		
All races						
Slow	162	29.0[d]	65	30.4[d]	227	29.5[e]
Rapid	91	25.6[d]	85	25.1[d]	176	25.3[e]
Total[d]	253	27.2[f]	150	27.5[f]		

[a] Adjusted for number of cigarette-equivalents smoked per day. The tobacco contents of one cigar and one pipe were assumed to be equivalent to the tobacco contents of 4.5 and 2.5 cigarettes, respectively.
[b] Further adjusted for *NAT1* genotype: $P = 0.12$, NAT2 slow *vs.* rapid.
[c] Further adjusted for NAT2 phenotype: $P = 0.99$, non-*NAT1*10 vs. NAT1*10*.
[d] Further adjusted for race (white, non-white).
[e] Further adjusted for race and *NAT1* genotype: $P = 0.02$, NAT2 slow *vs.* rapid.
[f] Further adjusted for race and NAT2 phenotype: $P = 0.86$, non-*NAT1*10 vs. NAT1*10*.

Discussion

Earlier, based on a data set that overlapped slightly with the current study (41 subjects were common to both sets), we reported that NAT2 slow acetylators consistently exhibited higher mean levels of 3- and 4-ABP-Hb adducts relative to rapid acetylators, independent of race (whites, blacks, Asians) and level of cigarette smoking (14). The present study validates those previous results. In all races combined, 3- and 4-ABP-Hb adduct levels were 47 and 16% higher in NAT2 slow *versus* rapid acetylators, respectively, after adjustment for race, smoking, and *NAT1*10* genotype. This finding is consistent with the overall evidence suggesting that NAT2 slow acetylators have an increased risk of bladder cancer (6).

This is the first study to investigate the impact of *NAT1* genotype on 3- and 4-ABP-Hb adduct levels in humans. We observed no independent influence of *NAT1*10* genotype on either 3- or 4-ABP-Hb adduct levels. Three factors potentially could contribute to the absence of a *NAT1*10* genotype effect.

First, despite early reports that *NAT1*10* was associated with higher than normal *p*-aminobenzoic acid enzyme activity in tissue samples (20), it remains unclear whether the *NAT1*10* allele is functionally related to differences in NAT1 enzyme activity (21, 22). Thus, the extent to which the *NAT1*10* polymorphism affects NAT1 phenotype is a currently unresolved issue.

Second, the impacts of the NAT1 enzyme on *N*-acetylation (detoxification) of ABPs on the one hand and on *O*-acetylation (bioactivation) of *N*-hydroxy-ABPs on the other hand may cancel each other out, resulting in the absence of an overall effect of NAT1 activity on 3- and 4-ABP-Hb adduct levels. For example, 4-ABP and *N*-hydroxy-4-ABP are better substrates for *N*- and *O*-acetylation, respectively, by NAT1 than NAT2 as evidenced by a clearance ratio >1 found in experiments expressing recombinant human NAT1 and NAT2 in different *Escherichia coli* strains (4). Thus, NAT1 could substantially impact the formation of ABP-Hb adducts via both *N*- and *O*-acetylation in the liver and extrahepatic tissue (including erythrocytes), respectively (17). In fact, even in the liver, which is the predominant site of aromatic amine detoxification via *N*-acetylation and where NAT1 expression generally is substantially lower than NAT2 expression, benzidine-exposed individuals with nonfunctional *NAT2* genes still form significant quantities of *N*-acetylated aromatic amines in their livers (23) and excrete *N*-acetylated aromatic amines in their urine (24).

Third, the effect of a functional polymorphism in *NAT1* on ABP-Hb adducts also depends on the pathway the chemical takes through the body, including the relative importance of metabolism in the liver *versus* metabolism in extrahepatic tissues such as the bladder or blood cells. Differences between the urothelium and the erythrocytes in expression of NAT1 and other metabolic enzymes and in the transport of ABP metabolites will determine whether ABP-Hb adducts are good biomarkers of ABP biotransformation in the bladder. Therefore, the lack of a *NAT1*10* effect on 3- and 4-ABP-Hb adduct levels may not be indicative of a lack of a *NAT1*10* effect on 3- and 4-ABP-DNA adduct levels in the urothelium, and consequently on bladder cancer risk.

In conclusion, our finding of a lack of a *NAT1*10* effect on 3- and 4-ABP-Hb adduct levels indicates that 3- and 4-ABP-Hb adduct levels are noninformative on the association of the *NAT1*10* genotype with bladder cancer risk. Our ongoing case-control study of bladder cancer will allow us to further address the relevance of 3- and 4-ABP-Hb adduct levels and of *NAT1*10* genotype to bladder cancer development.

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